Short title: Control of isometric gigantism in tomato

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Article title: The *ORGAN SIZE (ORG)* locus contributes to isometric gigantism in domesticated tomato

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One sentence summary: A locus that controls isometric size increase in vegetative and reproductive organs of tomato through changes in cell division

M.H.V performed the experiments using A.V.O.F. methods and analysed the data; C.R.F.F. provided technical assistance to M.H.V.; K.M. performed experiments using F.M. methods and analysed the data with assistance from Z.K.; A.Z. and A.J.T. analysed the data and wrote the article with contributions of all the authors; L.E.P.P

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36

37 **Abstract**

38 Gigantism is a key component of the domestication syndrome, a suite of traits that
39 differentiates crops from their wild relatives. Allometric gigantism is strongly marked in
40 horticultural crops, causing disproportionate increases in the size of edible parts such as
41 stems, leaves or fruits. Tomato (*Solanum lycopersicum*) has attracted attention as a
42 model for fruit gigantism, and many genes have been described controlling this trait.
43 However, the genetic basis of a corresponding increase in size of vegetative organs
44 contributing to isometric gigantism, has remained relatively unexplored. Here, we
45 identified a 0.4 Mbp region on chromosome 7 in introgression lines (ILs) from the wild
46 species *Solanum pennellii* in two different tomato genetic backgrounds (cv. M82 and
47 cv. Micro-Tom) that controls vegetative and reproductive organ size in tomato. The
48 locus, named *ORGAN SIZE* (*ORG*), was fine-mapped using genotype-by-sequencing. A
49 survey of literature revealed that *ORG* overlaps with previously mapped QTLs
50 controlling tomato fruit weight during domestication. Alleles from the wild species led
51 to reduced cell number in different organs, which was partially compensated by greater
52 cell expansion in leaves but not in fruits. The result was a proportional reduction in leaf,
53 flower and fruit size in the ILs harbouring the wild alleles. These findings suggest that
54 selection for large fruit during domestication also tends to select for increases in leaf
55 size by influencing cell division. Since leaf size is relevant for both source-sink balance
56 and crop adaptation to different environments, the discovery of *ORG* could allow fine-
57 tuning of these parameters.

58

59 **Introduction**

60

61 The domestication syndrome is the suite of phenotypic changes that occurred
62 through artificial selection to transform wild species into crops (Evans 1996). Some of
63 the most commonly found traits in crops are increased apical dominance, determinate
64 growth and loss of natural seed dispersal (Meyer et al. 2012; Denham et al. 2020). An

65 increase in the size of certain organs, or gigantism, is also widespread, particularly in
66 horticultural crops (Schwanitz 1957). Gigantism can be isometric, *i.e.* a proportional
67 increase in all body parts, but most generally occurs through allometric alterations in the
68 relative size of certain plant structures (Niklas 2004). A prime example is the species
69 *Brassica oleracea*, where multiple cultivated strains were produced through artificial
70 selection on the differential growth of edible organs such as stems (kohlrabi), buds
71 (cabbage, Brussels sprouts), leaves (kale) and flowers (broccoli, cauliflower) (Prakash
72 et al. 2011). Although increased organ size can be explained by alterations in cell
73 division and expansion (Krizek 2009), it also requires developmental alterations to
74 transform larger organs into stronger photosynthetic sources or sinks (Gifford et al
75 1984). Given that photosynthesis as a biochemical process has not been improved by
76 crop domestication or breeding (Orr et al. 2017; Batista-Silva et al. 2020), most of the
77 genetic gains in productivity have occurred indirectly through changes in plant
78 development (Greenland et al. 1997; Zsögön and Peres 2018).

79 In tomato (*Solanum lycopersicum* L.), gigantism is evidenced in the
80 phenomenal increase in fruit size when compared to its wild progenitor *S.*
81 *pimpinellifolium* (Tanksley 2004). The genetic basis of fruit size control has attracted
82 considerable attention (reviewed in Azzi *et al.*, 2015). Increased fruit size in tomato
83 involves mutations in multiple loci, some of which have been characterized at the
84 molecular level, for instance *fruit weight 2.2* (*fw2.2*), *fw3.2*, *fw11.3*, *fasciated* (*fas*),
85 *locule number* (*lc*) and *EXCESSIVE NUMBER OF FLORAL ORGANS* (*ENO*). All of
86 them are involved in fundamental processes of plant developmental such as cell
87 division, expansion and differentiation. The *FW2.2* gene is a negative regulator of cell
88 division responsible for up to 30% of the increase in fruit size when comparing lines
89 harbouring small- and big-fruit alleles (Frary et al. 2000). *FW3.2* and *FW11.3* were
90 identified as a P450 enzyme of the CYP78A subfamily (*SIKLUH*) and a *Cell Size*
91 *Regulator* (*CSR*), controlling cell division and expansion, respectively (Chakrabarti et
92 al. 2013; Mu et al. 2017). Unlike *fw2.2*, *fw3.2* and *fw11.3*, which mostly affect fruit size,
93 *fas* and *lc* also control fruit shape. The big-fruit *fas* and *lc* alleles increase the number of
94 carpels, altering cell differentiation through the CLAVATA3-WUSCHEL module
95 (Schoof et al. 2000). The increase in the number of carpels often results in larger and
96 wider fruits with many locules and pronounced ribbing (Lippman and Tanksley 2001;

van der Knaap and Tanksley 2003). The *lc* mutant phenotype is caused by two single-nucleotide polymorphisms (SNPs) downstream of the coding region of the *WUSCHEL* (*WUS*) gene (Muños et al., 2011). The *fas* mutation is a partial loss of expression caused by a chromosome inversion with a break point in the vicinity of the *CLAVATA3* (*CLV3*) gene (Xu et al 2015), a negative regulator of *WUS* (Schoof et al. 2000). Lastly, *ENO* is an AP2/ERF transcription factor that interacts synergistically with *lc* and *fas*, causing a substantial increase of the *WUS* expression domain, which results in enlarged floral meristems (Fernández-Lozano et al., 2015; Yuste-Lisbona et al., 2020). Thus, the *ENO* domestication allele (a promoter deletion that knocks down its expression) also affects stem cell fate, giving rise to multilocular fruits that derive from the larger floral meristem.

Compared with the genetic regulation of fruit growth, relatively little is known about the control of vegetative organ size. In many crops, including tomato (Supp Fig. S1) but also peppers (Jarret et al., 2019), sunflower (Warburton et al., 2017), soybeans (Kofsky et al., 2018) and common beans (Herron et al., 2020), domestication entailed the selection of plants with bigger shoots and leaves. In tomato, the proportional increase in the size of vegetative parts is likely to be a component of isometric gigantism during domestication. Herein, we hypothesized that if vegetative gigantism is under genetic control, the wild species' alleles leading to reduced organ size could be found through wide crosses between cultivated tomato and its wild relative species. We selected *S. pennellii* as a wild parental, due to its annotated genome sequence (Bolger et al. 2014) and its rich repertoire of genomic tools, such as fully sequenced introgression lines (Alseikh et al. 2013; Chitwood et al. 2014). We crossed it to the cultivated tomato cv. Micro-Tom (MT) and after successive backcrosses and phenotypic selection, we isolated an introgression line with reduced vegetative and reproductive organs compared to the recurrent parental MT. We mapped this introgression to chromosome 7 and named the locus *ORGAN SIZE* (*ORG*). We show that *ORG* leads to reduced organ size through changes in cell division, and that it segregates as a monogenic, semi-dominant Mendelian locus. Our fine mapping results show that the *ORG* candidate genes overlap a previously described domestication sweep (Lin et al. 2014). We speculate on the impact of this locus in the tomato domestication syndrome and discuss its potential exploitation for crop breeding.

129

130 **Results**

131

132 *Natural genetic variation for leaf size in tomato*

133 Compared to domesticated tomato cultivars, most wild relatives of the tomato
134 have small leaves (Supplemental Figure S1). Thus, we decided to look for a genetic
135 determinant of leaf size in the wild species. We crossed *S. pennellii* to the cultivated
136 tomato cv. Micro-Tom (MT). Upon self-fertilization of the F₁ population, we selected
137 F₂ plants with small leaves, from which we collected pollen to backcross (BC) to MT.
138 After six rounds of backcrossing to the recurrent parental (MT), self-fertilization
139 (BC₆F₂), phenotypic screening, and further self-fertilization (BC₆F_n), we produced an
140 introgression line (IL) with reduced leaf size in the MT background, which we called
141 *ORGAN SIZE (ORG)* (Figure 1). *ORG* plants show a very conspicuous phenotype for
142 leaf size: the difference in leaf size between MT and *ORG* was consistent across all
143 leaves and developmental stages (Figure 1). Monogenic segregation of *ORG* was
144 verified on a segregating population of MT and *ORG*. We determined leaf size in F₁
145 hybrids between MT and *ORG*, and the intermediate phenotype suggested that *ORG*
146 behaves as a semi-dominant gene (Supplemental Figure S2).

147

148 *The smaller leaf size in ORG is caused by reduced cell division*

149 Change in organ size is due to either altered cell proliferation or expansion, or a
150 combination of both (Krizek 2009). We analysed *ORG* leaves and found enlarged
151 epidermal and mesophyll cells compared to MT (Supplemental Figure S3). This
152 suggests that the smaller leaves of *ORG* are caused by reduced cell proliferation as
153 evidenced by cell number and density of *ORG* compared to MT (Supplemental Figure
154 S3). The greater palisade parenchyma cell size promoted an increase in leaf thickness in
155 *ORG*. We next performed a time course analysis of reproductive growth starting eight
156 days before anthesis and until 16 days after anthesis and verified a decrease in the size
157 of styles, ovaries and fruits in *ORG* (Figure 2). As in the case of leaves, the reduction
158 was caused by lower cell numbers, which we verified as a reduced number of cell layers
159 in the pericarp. The ovary cells of *ORG* were also smaller than MT cells at anthesis and
160 post-anthesis. Other floral organs, namely, petals and sepals, were also reduced in *ORG*

flowers compared to MT (Supplemental Figure S4). The reduced size of floral organs may have strong consequences on fruit development, given their impact on ovary size (Supplemental Figure S4e-h).

Fruit weight and yield are reduced in ORG

The size and shape of the ovary before anthesis is strongly correlated with the final size and shape of the fruit (Grandillo et al., 1999; Azzi et al., 2015). Thus, we next analysed the potential impact of *ORG* on fruit development. Fruit set was reduced in heterostylis *ORG* flowers, so we hand-pollinated emasculated MT and *ORG* flowers in a reciprocal cross. Several ovaries per plant were pollinated, but after fruit set confirmation (five days after pollination), we performed selective fruit removal to allow only five fruits to set on each plant. The presence of *ORG* ovaries had a substantial impact on the final fruit size regardless of pollen origin (Figure 3). Fruit weight was 31-37% lower in *ORG* than in MT ($P < 0.0001$, Supplemental Table S2). *ORG* fruits have higher total soluble solids content (°Brix) compared to MT (Supplemental Figure S5). We further observed that *ORG* had a similar frequency of locule number per fruit and reduced seed number (Supplemental Figure S5). Reciprocal crosses indicated that the reduction in seed number is determined by *ORG* ovaries rather than pollen (Supplemental Figure 5c).

We next addressed the possibility that reduced fruit size could be the consequence of altered photosynthetic source-sink relationships due to reduced leaf area. We thus manipulated the plants to maintain the availability of sources (leaves) constant and altered the source:sink ratio by changing the number of sinks (fruits). Three treatments were performed: either three, six or nine fruits were allowed to set on each plant. To ensure that additional sinks did not interfere in the results, we also pruned all the plants to remove side shoots. The results are summarized on Figure 3c-e. *ORG* plants produced consistently smaller fruits than MT in all treatments (Figure 3). The increase in fruit number, from three to six, promoted a reduction in fruit weight only in MT plants, suggesting that leaf area was a limiting factor to the final fruit weight in MT, since the leaf area was similar in both experimental conditions (Figure 3). On the other hand, when the number of fruits was increased from six to nine, there was a reduction in the final fruit weight for both genotypes. These results suggest that the

193 smaller leaf size of *ORG* could also account for its reduced fruit size, but only under full
194 fruit load. Therefore, the primary cause of the reduced fruit size in *ORG* is likely a
195 direct effect of this organ development since the pre-anthesis (Fig. 2c). In addition, the
196 presence of the *ORG* introgression reduced the yield in all treatments.

197

198 ***Expression patterns are altered in genes related to cell division and expansion in*** 199 ***ORG***

200 The results described so far suggest that the transcriptional activity of genes
201 involved in the control of cell division and expansion could be altered in *ORG*. To
202 assess this, we extracted mRNA from ovaries/fruits at -8, -4, 0, 4 and 8 days pre/post
203 anthesis, and fruit pericarps at 12 and 16 days to analyse the transcriptional profile of a
204 set of genes related to the control of cell division: *CYCLIN B2;1* (Solyc02g082820),
205 *FW2.2* (Solyc02g090730), *FW3.2/SIKLUH* (Solyc03g114940) and *EXPANSIN*
206 *PRECURSOR 5* (Solyc02g088100).

207 In ovary/fruit tissues, we verified that the mRNA levels of the cell-division
208 genes *CYCB2;1* and *FW3.2* showed greatest expression in both genotypes at 4 days pre-
209 anthesis (Figure 4). *CYCB2;1* was higher in MT than *ORG* especially in pre-anthesis
210 and anthesis stages (at -4, -8, and 0 days), while *FW3.2* was higher in anthesis and post-
211 anthesis stages (at 0, 4 and 12 days). On the other hand, *FW2.2*, another cell-division
212 gene, but a negative regulator, was highly expressed at 4 and 8 days post-anthesis in
213 both genotypes. Quantitative variation in *FW2.2* expression was observed pre- and post-
214 anthesis between genotypes (at -4 and 8 days, respectively), whereas *ORG* ovaries
215 showed significant increased levels of this transcript compared than MT (Figure 4).
216 After 4 days post-anthesis, the expression of the cell-expansion gene *EXPA5*, a member
217 of the α -expansin gene family, increased in in both genotypes (Figure 4). However,
218 ovaries of *ORG* plants displayed a significant decrease in the expression of this gene at
219 anthesis compared to MT. Similar behavior was observed 16 DPA.

220

221 ***The ORG locus is located on chromosome 7***

222 We next conducted a genotyping by sequencing (GBS) analysis to determine the
223 size and location of the *S. pennellii* introgression in *ORG*. The results show a discrete
224 region in the terminal end of the long arm of chromosome 7 encompassing ~11 Mb

(Figure 5). No further segments of *S. pennellii* genome were found on other chromosomes. Based on the SL2.50 tomato genome annotation, the introgression region contains 1169 genes. A closer look at the introgressed region revealed a small double recombination, from position 64,826,717 to 65,444,176, encompassing 78 tomato genes which score as *S. lycopersicum* (Figure 5b).

Fine-mapping of ORG using introgression lines

To reduce the list of candidate genes for *ORG*, we next analysed two other introgression lines (ILs) of *S. pennellii* in the MT background previously generated in our laboratory: *Brilliant corolla* (*Bco*) and *Regeneration 7H* (*Rg7H*), both of which partially overlap either end of the *ORG* introgression (Figure 6). We used the span of the introgressions in *Bco* and *Rg7H* and the extent of their overlap with *ORG* (Supplemental Figure 6 for *Bco* and Pinto *et al.*, 2017 for *Rg7H*) to narrow down the candidate region for *ORG*. Given that neither of these ILs show the reduced organ phenotype of *ORG*, the resulting candidate region is located between positions 65,444,176 and 66,373,175 (Figure 6a).

We took advantage of the existing collection of ILs from *S. pennellii* in tomato cv. M82 as a tool to further refine the above chromosome location (Zamir and Eshed 1994; 1995). The introgressions were precisely delimited by sequencing by Chitwood *et al.* (2014), who also characterized terminal and lateral leaflet size in the ILs. Their results revealed the existence of a QTL for reduced leaflet size on both IL7-2 and IL7-3 (Figure 7a-b). We also cultivated ILs harbouring *S. pennellii* genomic segments on chromosome 7 (IL7-1; IL7-2, IL7-3; IL7-4 and IL7-5) and determined their leaf and ovary size. We found a reduction in the ovaries of both IL7-2 and IL7-3, compared to M82, but under our growth conditions only IL7-2 showed consistently smaller leaves than the parental line (Supplemental Figure S7). We found a discrepancy between the Chitwood *et al.* dataset and ours for leaf size on IL7-1, but the consistently smaller pistils in IL7-2 and IL7-3 helped us delimit the right border of the candidate region to 65,865,655, narrowing the interval to 421,479 bp (Figure 7c).

Genomic analysis of ORG and identification of candidate genes

256 The resequenced dataset of tomato and wild relative accessions (Aflitos et al.,
257 2014) was used to identify the polymorphisms of *S. pennellii* when aligned with *S.*
258 *lycopersicum* (SL2.50) in the *ORG* region. We found 58 CDS within the *ORG* region in
259 the *S. pennellii* genome and 65 CDS within *S. lycopersicum*, with considerable synteny
260 (Figure 7d). Within the *ORG* region, an alignment of the *S. pennellii* genome sequence
261 (Spenn-ch07:76,477,056-76,940,423) with *S. lycopersicum* (SL2.50ch07:65,444,176-
262 65,865,655) showed that the two genomes were structurally similar (Supplemental
263 Figure S8). We therefore investigated the similarities and differences in the coding
264 sequences (CDS) between the two genomes with BLAST (Supplemental Table S3). We
265 found a total of 6,009 polymorphisms, 5,093 of which were single-nucleotide
266 polymorphisms (SNPs) and 916 were insertions-deletions (InDels). Additionally, there
267 were 304 moderate effect missense variants affecting 58 genes (Supplemental Table S4)
268 and 18 high effect polymorphisms (*e.g.* frameshift variants, stop gained) (Supplemental
269 Table S5). There was one *S. pennellii* CDS without a corresponding match in *S.*
270 *lycopersicum*, *i.e.* a new gene within the *ORG* region, namely Sopen07g031050
271 (hypothetical protein). Additionally, there were six presence-absence variants (PAVs) in
272 *S. lycopersicum* without a corresponding match in *S. pennellii* (Supplemental Table S6),
273 *i.e.* six genes lost in the *ORG* region, namely, a Yippee family protein
274 (Soly07g062900), a nucleolar GTP-binding protein 2 (Soly07g063280), a Tir 2C
275 resistance protein (Soly07g063360) and three CDS annotated as ‘unknown protein’.
276 The genes Sopen07g031090 and Sopen07g031100, both being putative Yippee family
277 zinc-binding proteins, produced multiple significant matches with Soly07g062880,
278 Soly07g062890 and Soly07g062910. Additionally, Sopen07g031530 (beta
279 glucosidase 46) and Sopen07g031540 (hypothetical protein) produced only partial
280 matches with Soly07g063370 (beta glucosidase) and Soly07g063380 (unknown
281 protein), respectively; indicating that the gene pairs share conserved regions but are
282 otherwise dissimilar (Figure 7d).

283 Discussion

284 The genetic basis of fruit gigantism has been extensively explored in tomato and
285 a number of major genes controlling that trait have been identified (Nesbitt and
286 Tanksley 2001; Causse et al. 2004; Muñoz et al. 2011; Chakrabarti et al. 2013; Mu et al.
287 2017). However, the genetic mechanisms behind isometric gigantism between

288 vegetative and reproductive organs are unknown. Are they driven pleiotropically by
 289 genes for fruit gigantism that operate on the meristem simultaneously controlling
 290 vegetative and reproductive development, or are they the product of indirect selection
 291 on independent loci necessitated by the altered source-sink relationships between
 292 vegetative or reproductive organs? As a starting point to address this question, we set
 293 out to discover genetic determinants for changes in the size of vegetative organs in the
 294 tomato. We thus identified *ORGAN SIZE (ORG)*, an introgression with reduced leaf size
 295 but which also showed smaller reproductive organs, namely flowers and fruits.

296 Instead of the conventional approach of QTL mapping, which sometimes is
 297 followed by fine-mapping and gene cloning, we revisited the alternative, forward
 298 genetics strategy, of wide cross followed by controlled introgression (Rick, 1969). We
 299 crossed *S. pennellii* to the tomato cv. Micro-Tom (MT) and conducted multiple rounds
 300 of crosses and backcrosses to the recurrent domesticated parental, selecting plants with
 301 smaller leaves in each generation. Our results, which identified the *ORG* locus, tie up
 302 previous, independent studies of the genetic control of leaf (Holtan and Hake, 2003;
 303 Chitwood *et al.*, 2014) and fruit (Grandillo *et al.* 1999; van der Knaap and Tanksley
 304 2003; Causse *et al.* 2004; Barrantes *et al.* 2016) size in tomato using QTL analysis.
 305 Hence, a survey of previous studies that identified putative QTLs for increased fruit
 306 weight during tomato domestication and breeding reveal a chromosomal region
 307 overlapping *ORG* (Supplemental Figure 9). However, none of these studies reported
 308 alterations in vegetative development associated to fruit weight QTLs. This indicates
 309 that controlled introgression guided by phenotypic selection is a powerful tool that,
 310 unlike QTL mapping, allows the detection of genes (or closely linked genes) that
 311 control more than one trait simultaneously. Either QTL mapping, or its more up-to-date
 312 variant, genome-wide sequencing analysis (GWAS), are useful to detect multiple genes
 313 spread out in the genome controlling one trait, but on the other hand, are prone to miss
 314 pleiotropic or tightly linked genes controlling multiple traits, because generally only one
 315 phenotype is analysed at a time (Korte and Farlow, 2013).

316 Genotyping-by-sequencing showed *ORG* to harbour 1169 genes in
 317 approximately 11 Mb of *S. pennellii* genome. This represents 1.15% of the tomato
 318 genome, which is a good fit with the theoretically expected proportion of donor genome
 319 after six rounds of back-crossing (Stam and Zeven 1981). Although the segregation data

indicate that *ORG* behaves as a Mendelian, semi-dominant gene, we cannot at this stage exclude the possibility that the IL harbours two or more genes controlling similar traits on chromosome 7. However, we showed that the common denominator for the reduced size of vegetative and reproductive organs in *ORG* is a reduction in the number of cells, possibly through alteration of cell division rate, as suggested by our gene expression analyses for *CYCB2;1*, *FW2.2* and *FW3.2*. This trait could be under pleiotropic control of a single gene. In fact, our analysis of the genes contained in the candidate region shows variation between *S. pennellii* and *S. lycopersicum* for genes predicted to be involved in the control of cell division, as well as regulatory genes that could control the size of organs (Supplemental Tables S4 and S5). An interval containing 19 putative domestication genes was also identified on chromosome 7 by Lin *et al.* (2014) by analyzing the genome sequence of 360 tomato accessions. All 19 genes are contained within the list of 58 candidates for the *ORG* region. This paves the way for the future identification and validation of, potentially, a single gene with a unique underlying variant (*e.g.* SNP, InDel, PAV) controlling organ size.

Increased organ size, or gigantism, is a recurrent domestication trait observed in many crops. Selection for increased size of edible parts led to allometric increases in reproductive organs. However, domesticated plants also tend to present gigantism in vegetative parts, *e.g.* larger leaves and thicker stems in *Phaseolus vulgaris* (Donald and Hamblin, 1983), larger leaves in eggplant (Page *et al.*, 2019) and soybean (Kofsky *et al.*, 2018). The tomato shows striking increases in fruit size (Tanksley 2004), but also leaf area, and stem thickness compared to its wild relatives (Milla and Matesanz, 2017). This isometric size change could lead to a better balance between photosynthetic sources and fruit sinks. When we altered the relative strength of the sinks by allowing only three, six or nine fruits to develop in either MT or *ORG* plants, we found an inverse correlation between fruit number and size in MT but not in *ORG*. In addition, the reduction in fruit size of MT has no penalty in its final yield. These results suggest two things. First, that the reduced size of *ORG* fruits is an intrinsic trait, possibly a developmental result of smaller ovaries, and not an indirect consequence of reduced leaf area (photosynthetic source). The second is that leaf area is not always directly limiting fruit (sink) size and/or yield. In agreement with this, both experimental and modelling work have shown that defoliation does not have a negative effect on crop yield,

implying that source strength is not limiting (provided water and nutrient availability are sufficient and that photosynthesis is not light limited) (Heuvelink *et al.*, 2005). An extreme situation is found in garden peas (*Pisum sativum*), where leaf area reduction has been a breeding goal to reduce interplant competition and increase yield (Cousin, 1997). Mutants of the ‘leafless’ and ‘semi-leafless’ type show 40% lower leaf area with up to 20% higher yield and better standing ability, which in turn facilitates mechanical harvesting (Checa *et al.*, 2020). The increased popularity and growing market niche for ‘gourmet’ cherry tomatoes opens up the perspective of breeding varieties with smaller leaves to improve agronomic management (*e.g.* reduced fertilizer, water use) (Sarlikioti *et al.*, 2011).

362

363 **Conclusions**

Based on the analysis of natural genetic variation, we have described a potential genetic determinant for increased leaf size in cultivated tomato. Our results could unveil a novel link in the genetic control of isometric fruit and leaf gigantism in tomato. Further research to determine the molecular identity of the gene(s) underlying the *ORG* phenotype is underway. This knowledge would be a valuable addition in the repertoire of gene targets that can be manipulated with ideotype breeding (Donald, 1968; Zsögön *et al.* 2017) or *de novo* domestication platforms (Gasparini *et al.*, 2021).

371

372 **Materials and methods**

373

374 *Plant material*

The wild relatives of tomato used in this work were *S. pennellii* (LA0716), *S. chilense* (LA1969), *S. peruvianum* (LA1537), *S. neorickii* (LA1322), *S. chmieslewskii* (LA1028), *S. habrochaites* f. *glabratum* (PI134417), *S. habrochaites* f. *hirsutum* (LA1777), *S. galapagense* (LA1401), *S. pimpinellifolium* (CNPH384), and *S. lycopersicum* var. *cerasiforme* (LA1320). Domesticated tomatoes of the cultivars Micro-Tom (MT) (LA3911), M82 (LA3475), Moneymaker (LA2706) and Santa Clara (Brazilian local cultivar) were also used. The *S. pennellii* chromosome 7 introgression lines (ILs) harboring alleles of *ORGAN SIZE* (*ORG*), *BRILLIANT COROLLA* (*Bco*) (Chetelat 1998) and *Rg7H* (Pinto *et al.* 2017) were obtained through repeated backcrossing

384 between cultivated MT as a pollen receptor and *S. pennellii*, as described in Carvalho et
385 al. (2011). Seeds of the tomato wild relatives were obtained from the UC Davis/C.M.
386 Rick Tomato Genetics Resource Center, maintained by the Department of Plant
387 Sciences, University of California, Davis, CA 95616. Seeds of MT were kindly donated
388 by Prof. Avram Levy (Weizmann Institute of Science, Israel) in 1998 and kept as a true-
389 to-type cultivar through self-pollination.

390 *Growth conditions*

391 Plants were grown in a greenhouse at the Laboratory of Plant Developmental
392 Genetics, ESALQ-USP, (543 m a.s.l., 22° 42' 36" S; 47° 37' 50" W), Piracicaba, SP,
393 Brazil. Automatic irrigation took place four times a day. Growth conditions were: mean
394 temperature of 28°C, 11.5 h/13 h (winter/summer) photoperiod, 250–350 $\mu\text{mol photons}$
395 $\text{m}^{-2} \text{s}^{-1}$ PAR irradiance, attained by a reflecting mesh (Aluminet, Polysack Indústrias
396 Ltda, Leme, Brazil). Seeds were germinated in 350 mL pots with a 1:1 mixture of
397 commercial potting mix Basaplant® (Base Agro, Artur Nogueira, SP, Brazil) and
398 expanded vermiculite supplemented with 1 g L⁻¹ 10:10:10 NPK and 4 g L⁻¹ dolomite
399 limestone (MgCO₃ + CaCO₃). Upon the appearance of the first true leaf, seedlings were
400 transplanted to pots containing the soil mix described above, except for NPK
401 supplementation, which was increased to 8 g L⁻¹. In addition, MT and OS plants
402 received a supplementary fertilization of 0.5g of NPK formulation 10:10:10 after
403 flowering. Cultivated and wild tomato plants were supplemented with 2g of NPK
404 formulation 10:10:10 per plant.

406 *Phenotypic characterization*

407 We scanned all leaves of the MT and *ORG* plants 40 days after germination
408 (dag) and determined the leaf area using ImageJ software (<http://rsbweb.nih.gov/ij/>).

409 For the characterization of floral whorls, we evaluated: length of petals and
410 sepals; corolla area; and ovary weight, height and diameter. To measure ovary length
411 and height we used a magnifying glass (Leica S8AP0, Wetzlar, Germany), coupled to a
412 camera (Leica DFC295 Wetzlar, Germany). To determine ovary weight we determined
413 the weight of 1.5 mL Eppendorf microtubes with 1 mL of distilled water, before and
414 after collection of 10 ovaries. Ovary weight was then determined as the difference
415 between initial and final tube weight. We also evaluated the leaf area and ovary weight

of M82 plants and introgression lines (ILs) from chromosome 7, using the same methodology as for MT and *ORG*.

MT and ORG productivity traits

We hand-pollinated MT and *ORG* plants with pollen from MT and *ORG* plants, because the *ORG* genotype displayed low fruit set. Various ovaries were pollinated, but after fruit set confirmation (five days after pollination), we performed selective fruit removal to allow only five fruits to set on each plant.

Productive performance of plants was assessed 90 days after germination. The following parameters were determined: mean weight per fruit; total soluble solids content in fruits (Brix); locule number and number of seeds per fruit; and weight of 10 seeds. Total soluble solids content of fruits was assessed using a digital refractometer (PR-101, Atago, Tokyo, Japan).

Source-sink ratio in MT and ORG plants

To determine whether leaf area of *ORG* plants is a limiting factor for fruit development (since leaves and fruits are the major sources and sinks of photoassimilates, respectively), we manipulated plants creating three categories based on different source-to-sink ratios. Thus, we kept the same amount of source tissue (leaves) in all plants of each genotype and altered the sink strength by changing fruit number (either three, six or nine per plant, to produce high, medium or low source-to-sink ratios, respectively). We removed side branches to prevent them from acting as alternative sinks. The following parameters were then determined: total fruit weight per plant (yield); average fruit weight and whole-plant leaf area.

Mapping and PCR amplification of DNA markers

We designed molecular markers to discover polymorphisms between tomato and *S. pennellii* in the region comprising the IL-7-2 and part of the IL 7-4 (Chitwood et al., 2014). The sequences and types of molecular makers are shown on Supplemental Table S1. Two further genotypes harbouring genome segments of *S. pennellii* for chromosome 7, *Brilliant corolla* (*Bco*) and *Regeneration 7h* (*Rg7H*), both in cv. MT, were characterized molecularly and phenotypically. Cross-referencing information from these

448 genotypes and the ILs in the M82 background we constructed a map with the putative
449 location of the *ORG* locus.

450 Genomic DNA extraction from young leaves was performed as described by
451 Fulton et al. (1995). PCR was performed using the following program: a denaturation
452 step at 95°C for 2 min, 35 cycles of 30 s at 95°C, 60s at 56°C, 90 s at 72°C, and a final
453 cycle at 72°C for 7 min. When required, restriction enzyme analysis (Supplemental
454 Table S1) was performed following the manufacturer's recommendations (NEB,
455 Bethesda, USA). The final PCR products were analyzed via 1.5% (m/v) agarose gel
456 electrophoresis, stained with SYBR Gold (Invitrogen).

457

458 *Histological and microscopic analyses*

459 Samples of MT and *ORG* ovaries/fruits at -8, -4, 0, 4 and 8 days, and fruit
460 pericarps at 12 and 16 days (anthesis=0), were collected and fixed in Karnovsky
461 solution (Karnovsky 1965), and vacuum-infiltrated for 15 min. The times referred to as
462 -8 and -4 days correspond to 8 and 4 days before anthesis, respectively. We based these
463 on the length of the closed flower buds (Faria 2014).

464 Samples were next dehydrated in an increasing ethanol series (10–100%), and
465 infiltrated into synthetic resin, using a HistoResin embedding kit (Leica, [www.leica-](http://www.leica-microsystems.com)
466 [microsystems.com](http://www.leica-microsystems.com)), according to the manufacturer's instructions. The tissues were
467 sliced using a rotary microtome (Leica RM 2045, Wetzlar, Germany), stained with
468 toluidine blue 0.05% (Sakai 1973), and photographed in a microscope (Leica DMLB,
469 Heidelberg, Germany), coupled to a Leica DFC310 camera (Wetzlar, Germany).
470 Histological analysis of ovaries was performed in the central region of the outer
471 pericarp of the fruits, and the area and number of cells were determined using ImageJ
472 software (<http://rsbweb.nih.gov/ij/>). This histological analysis also was performed in the
473 mature leaves of these genotypes adopting the procedures described above. The area
474 and number of cells in the adaxial leaf epidermis of the MT and *ORG* genotypes was
475 also evaluated using the leaf dental resin imprinting technique (Weyers and Johansen
476 1985).

477

478 *Quantitative real-time reverse transcription PCR*

479 Total RNA was extracted from ovaries/fruits at -8, -4, 0, 4 and 8 days, and fruit
480 pericarps at 12 and 16 days (anthesis = 0), using Trizol reagent (Invitrogen), as
481 indicated by the manufacturer, and treated with RQ1 RNase-Free DNase (Promega).
482 Fruit pericarps were carefully collected from the central region of the outer pericarp of
483 the fruits, at 12 and 16 days. After DNase treatment, a single-strand cDNA was
484 synthesized from total RNA (1µg) by reverse-transcription, using RevertAid RT
485 Reverse Transcription Kit (Thermo Fisher Scientific).

486 Gene expression analyses were performed on a Rotor-Gene Q real-time PCR
487 cycler (Qiagen), using Kapa Sybr Fast qPCR Master Mix (Kapa Biosystems) and
488 specific primers for *CYCB2;1* (Soly02g082820), *FW2.2* (Soly02g090730), *FW3.2*
489 (Soly03g114940) and *EXP5* (Soly02g088100) genes. The reactions were amplified
490 for 2 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The
491 threshold cycle (C_T) was determined. Melting curve analysis was performed with each
492 primer set to confirm the presence of only a single peak before the gene expression
493 analyses. Two technical replicates were analyzed for each of three or four biological
494 samples. The relative transcript accumulation was normalized to an *ACTIN*
495 (Soly04g011500) gene. The fold changes for each gene were calculated using the
496 equation $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen 2001). Primer sequences used to qRT-PCR are
497 shown in Supplemental Table S1.

498

499 *In silico analysis of probable ORG region*

500 The genomes of *S. lycopersicum* cv. Heinz 1706, SL2.50
501 (<https://solgenomics.net/>) and *S. pennellii* LA716 (Bolger et al., 2014b) were aligned
502 and plotted with Mummer v4.0.0 (Marcais et al., 2018). Variants of *S. pennellii* LA716
503 versus SL2.50 within the *OS* region were obtained through the Wageningen
504 resequencing project (Aflitos et al., 2014). The coding sequences of the genes within the
505 region were obtained from Solanaceae Genomics Network (<https://solgenomics.net/>)
506 and similarities between Heinz 1706 and LA716 were tested with BLAST v2.10.0
507 (Camacho et al., 2009). The Circos plot was created with Circos v0.69.9 (Krzywinski et
508 al., 2009) on Windows 10. The synteny plot was created with the genoPlotR package
509 (Guy et al., 2011) within R (Team, 2017).

510 *Genotyping by sequencing (GBS)*

511 DNA was extracted from young leaf samples (~10 mm length) that were freeze-
512 dried (CoolSafe™ 55-9; Scanvac, Lynge, Denmark) overnight. Leaf samples were
513 powdered in a Star-Beater (VWR, Lutterworth, UK) at 30 Hz for 30s in 2 mL
514 microcentrifuge tubes containing two 5 mm acid-rinsed soda-glass balls. DNA was
515 extracted from ~50 mg samples with an E.Z.N.A® Plant DNA Kit (VWR, Lutterworth,
516 UK). DNA fragment size was assessed on a 1% agarose gel in Tris/Borate/EDTA to
517 confirm that all samples had the majority of DNA fragments >10 kilobases.

518 The GBS library was prepared using the restriction enzyme *MseI* and sequenced
519 on an Illumina NextSeq 500 V2 by LGC Genomics (Berlin, Germany). The 150 base-
520 pair paired-end reads were aligned to the *Solanum lycopersicum* Heinz 1706 reference
521 genome (SL2.50) with BWA v0.7.15 (Li and Durbin, 2009). The SAM files were
522 processed with Samtools Fixmate v1.3.1 (Li et al., 2009). InDels were realigned with
523 GATK's IndelRealigner v3.8-0 (McKenna et al., 2010; Depristo et al., 2011) before
524 variant calling with Samtools Mpileup v1.3.1 and Bcftools Call v1.3 (Li, 2011).

525 The raw VCF files of the GBS sample, a 40× resequenced Micro-Tom
526 (Cranfield University, unpublished data) and the resequencing of *S. pennellii* LA716
527 (Aflitos et al., 2014), were combined into an index using Tersect (Kurowski and
528 Mohareb, 2020). Tersect was used to determine which variants were shared between the
529 *ORG* IL and *S. pennellii* LA716, excluding the variants shared with Micro-Tom. The
530 variants output from Tersect were then filtered as follows: all variants with a quality
531 score less than 20, a mapping quality score below 40 and a raw read depth either below
532 10 and above 200 were removed. In addition, heterozygous variants were removed. The
533 variant density of the filtered variants over a 10 kb window (5 kb sliding) were plotted
534 across all 12 chromosomes with ggplot2 (Wickham, 2016) within R.

535

536 *Statistical analysis*

537 Statistical analysis was performed using SAS software (SAS Institute Inc., Cary,
538 NC, USA). The variables data were submitted to analysis of variance (ANOVA) and the
539 means compared by the Student's t- or Tukey's test. When the data did not meet the
540 assumptions of ANOVA, we performed to non-parametric analysis, using Wilcoxon
541 rank sum or Dunn's test to compare the means.

542

543 **Supplemental Data**

544 Supplemental Figure S1. Leaf size increases during tomato domestication and
545 improvement.

546 Supplemental Figure S2. Heterozygous *ORG* plants (*ORG*+) show an intermediate leaf
547 area compared to MT and *ORG* plants.

548 Supplemental Figure S3. Smaller leaf size in *ORG* is caused by reduced cell division.

549 Supplemental Figure S4. *ORG* reduces organ size in all floral whorls

550 Supplemental Figure 5. Fruit traits are altered in *ORG* plants.

551 Supplemental Figure 6. GBS defines the span of the introgression in the *Brilliant*
552 *corolla* (*Bco*) introgression line.

553 Supplemental Figure 7. Characterization of *S. pennellii* introgression lines (IL) in
554 chromosome 7.

555 Supplemental Figure 8. Alignment plot of the *S. pennellii* and *S. lycopersicum* genomes
556 within the *ORG* region.

557 Supplemental Figure 9. Colocalization of *ORG* and previously mapped fruit size QTLs.

558 Supplemental Table S1. Oligonucleotide sequences used for genotyping and
559 quantitative PCR analyses in this work.

560 Supplemental Table S2. Fruit weight of MT and *ORG* plants.

561 Supplemental Table S3. Similarities and discrepancies between coding sequences of *S.*
562 *pennellii* v. *S. lycopersicum* candidate genes.

563 Supplemental Table S4. Polymorphisms with a moderate effect on gene function for *S.*
564 *pennellii* v *S. lycopersicum* within the *ORG* region.

565 Supplemental Table S5. Polymorphisms with a high effect on gene function for *S.*
566 *pennellii* v *S. lycopersicum* within the *ORG* region.

567 Supplemental Table S6. Coding sequences of *S. lycopersicum* not producing a match on
568 the *S. pennellii* genome assembly.

569

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752

753 Figure legends

754

755 **Figure 1. A tomato introgression line (IL) from *S. pennellii* with reduced vegetative organs**
756 **(*ORG*) size. (a)** Crossing scheme to create an introgression line with smaller leaves in the
757 tomato cv Micro-Tom (MT) background **(b)** Representative population of MT (left) and *ORG*
758 (right) plants, 25 days after germination (dag). **(c)** Side and top view of MT (top) and *ORG*
759 (bottom) plants. **(d)** Leaf series of MT (top) and *ORG* (bottom) genotypes from cotyledons (C1)
760 to fifth leaf (L5). Scale bar=5 cm. **(e)** Leaf area of the leaf series of MT (gray bar) and *ORG*
761 (white bar) plants, 40 dag. Data are mean $\pm$ s.e.m. (n=14 leaves). Statistical significance was
762 tested by Student's *t*-test (***p*<0.001).

763

764 **Figure 2. *ORG* affects cell number and size during fruit development. (a)** Developing
765 ovary/fruit at -12, -8, -4, 0, 4, 8, 12 and 16 days (anthesis = 0). MT (top) and *ORG* (bottom).
766 Scale bar=5mm. **(b)** Longitudinal sections of MT (top) and *ORG* (bottom) pericarp at -12, -8, -
767 4, 0, 4, 8, 12 and 16 days (anthesis = 0). Scale bar = 150 μ m. **(c)** Time course of the number of

cell layers in the longitudinal sections of MT (gray bar) and *ORG* (white bar) ovary/fruit pericarp. Insert in top of this figure represents how the counting of the cells was performed and red lines delimited cell perimeter (n=30). (d) Time course of cell area in the cell layers of MT (gray bar) and *ORG* (white bar) (n=30). Data are mean $\pm$ s.e.m. Statistical significance was tested by Student *t*-test (* $p < 0.05$, *** $p < 0.001$, ns indicates non-significant differences).

Figure 3. Fruit growth and source-sink relationships are altered in *ORG*. (a) Representative MT (♀, left) and *ORG* (♀, right) ripe fruits pollinated with MT (♂, left) and *ORG* (♂, right) pollen. Scale bar=1 cm. (b) Mean (red) and median (black) values of fruit weight of MT (gray box) and *ORG* (white box) ripe fruits pollinated with MT (n=10) and *ORG* (n=14) pollen. (c) Frequency of locule number per fruit in MT and *ORG* fruits (n=125). (d) Seeds per fruit of MT and *ORG* pollinated with MT (n=11) and *ORG* (n=15) pollen. Data are mean $\pm$ s.e.m. Statistical significance was tested by Student's *t*-test (*** $p < 0.001$). (e-g) Average values of fruits weight (e), leaf area (f) and yield (g) from MT (gray bar) and *ORG* (white bar) plants pruned to three, six and nine fruits (n=6 plants per treatment). Data are mean $\pm$ s.e.m. Different capital and lowercase letters on the symbols indicate significant differences by Tukey's test ($p < 0.001$) between the treatments in MT and *ORG* genotypes, respectively.

Figure 4. Altered patterns of gene expression in *ORG*. Time course of transcript levels of cell division- and expansion-related genes in ovaries/fruits of MT (gray bar) and *ORG* (white bar) genotypes. Relative (to actin control) transcript levels of *CYCB2;1* (a), *FW2.2* (b), *FW3.2* (c) and *EXP5* (d) in ovaries/fruit at -8, -4, 0, 4, 8 days and fruit pericarp at 12 and 16 days (anthesis = 0). Data are mean $\pm$ s.e.m (n=3 biological replicates indicated with black dots). Statistical significance was tested by Student's *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 5. GBS defines the span of the introgression in the *ORG* introgression line (IL). (a) Genome-wide density of unique variants shared between *ORG* and *S. pennellii* LA716 in the genetic background of tomato cv Micro-Tom. (b) Close up view of the introgression on chromosome 7.

Figure 6. Mapping refines the candidate region for *ORG*. (a) Two introgression lines (ILs) in the tomato cv Micro-Tom (MT) background that contain different segments from *S. pennellii* on chromosome 7 (*Bco* and *Rg7H*) were mapped to refine the candidate region harboring the *ORG* locus (red segment). (b) Representative leaf of MT, *ORG*, *Rg7H* and *Bco* genotypes. Scale bar = 5 cm. (c-d) Leaf area (c) and ovary weight (d) of MT, *ORG*, *Rg7H* and *Bco* (n=10). Statistical significance was tested by Tukey's test ($p < 0.05$). Different letters indicate significant difference between genotypes.

Figure 7. Analysis of the genomic region containing *ORG*. Terminal (a) and lateral (b) leaflet area of M82 and chromosome 7 introgression lines (ILs) from *S. pennellii*. Statistical significance was tested by ANOVA followed by Tukey's HSD test. Redrawn from Chitwood et al. (2014). (c) Chromosomal position of *S. pennellii* genomes segments in tomato cv. M82 background in chromosome 7. The location of the *ORG* candidate region is shown in red. (d) Synteny plot of the coding sequences (CDS) within the *ORG* region between *S. lycopersicum* and *S. pennellii* genomes. The similarity between the CDS of *S. lycopersicum* (SL2.50) and *S. pennellii* (Spenn) were tested with BLAST+ and variant effect prediction was obtained from the resequenced dataset (Aflitos et al. 2014). Key: Dark green, CDS that match with a high level of similarity, but *S. pennellii* alleles contain single nucleotide polymorphisms (SNPs). Light green, *S. pennellii* alleles contain insertions and deletions (InDels). Red, *S. pennellii* alleles contain variants predicted to cause loss of function. Blue, complex relationship between *S. lycopersicum* and *S. pennellii* alleles, i.e. multiple matches between different genes. Grey, partial matches

819 between *S. lycopersicum* and *S. pennellii* alleles, *i.e.* CDS with conserved regions but otherwise
820 dissimilar. Black, genes present in *S. lycopersicum* or *S. pennellii* only.